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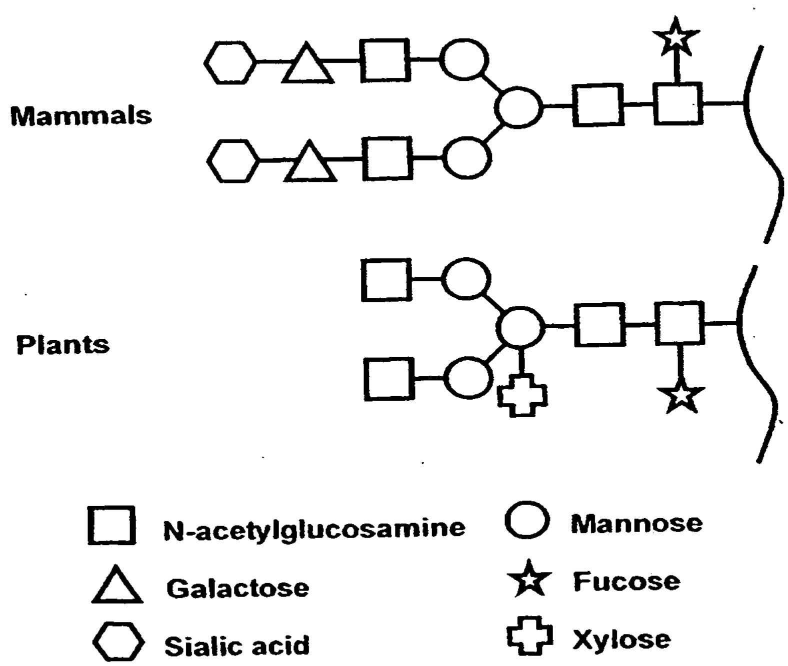
(54) Title: MAMMALIAN-TYPE GLYCOSYLATION IN PLANTS



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(57) Abstract: The invention relates to the field of glycoprotein processing in transgenic plants used as cost efficient and contamination safe factories for the production of recombinant biopharmaceutical proteins or pharmaceutical compositions comprising these. The invention provides a plant comprising a functional mammalian enzyme providing N-glycan biosynthesis that is normally not present in plants, said plant additionally comprising at least a second mammalian protein or functional fragment thereof that is normally not present in plants.

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Title: Mammalian-type glycosylation in plants.

The invention relates to the field of glycoprotein processing in transgenic plants used as cost efficient and contamination safe factories for the production of useful proteinaceous substances such as recombinant biopharmaceutical proteins or (pharmaceutical) compositions comprising these.
The creation of recombinant proteins as e.g. medicaments or pharmaceutical compositions by pharmaco-molecular agriculture constitutes one of the principal attractions of transgenic plants; it is also the domain where their utilization is accepted best by the public opinion. In addition to the yield and the favourable cost which may be expected from the field production of recombinant proteins, transgenic plants present certain advantages over other production systems, such as bacteria, yeasts, and animal cells. Indeed, they are devoid of virus which might be dangerous to humans, and can accumulate the proteins of interest in their "organs of storage", such as seeds or tubers. This facilitates their handling, their transportation and their storage at ambient temperature, while affording the possibility of subsequent extraction according to needs. Moreover, the transgenic plant, or some of its parts, can be utilised as vector of medicaments or of vaccines. In 1996, the team of Charles Arntzen (Boyce Thompson Institute for Plant Research, Cornell University, New York) has demonstrated the production of a recombinant vaccine against the thermolabile enterotoxin of *Escherichia coli* by the potato. Its efficacy has been demonstrated in mice and through clinical trials carried out on volunteers having consumed 50 to 100 grams of raw transgenic potatoes over a period of six months. Another team, at Loma Linda, in California, has successfully tested in mice a vaccine against cholera formed in the potato. Traditional vaccination against germs responsible for enteropathies is regarded as "too costly" to be generally implemented in developing countries. However, the production

of oral vaccines for example no longer in the potato but in the banana, would, at a very low cost, enable general implementation of vaccination against diarrheas of bacterial origin, which cause the death of three million children every year. In the developed countries, one can imagine that children would certainly prefer a banana or strawberry vaccine to the doctor's needle. More generally, molecular pharming could enable developing countries to produce, at low cost, substantial quantities of therapeutic proteins utilizing the capacities of their agriculture, without it being necessary to invest in pharmaceutical factories. Although the advantages of plants as factories of proteinaceous substances are explained mostly in the light of biopharmaceuticals, plants are also useful for production of other proteins, e.g. industrial enzymes and the like, because of their capability of glycosylation leading e.g. to higher stability. Today, the utilisation of plants for the production of proteins or glycoproteins for therapeutic use has gone widely beyond the domain of science fiction since soy, tobacco, the potato, rice or rapeseed is the object of investigations for the production of vaccines, proteins or peptides of mammals such as: monoclonal antibodies, vaccine antigens, enzymes such as canine gastric lipase, cytokines such as epidermal growth factor, interleukins 2 and 4, erythropoietin, encephalins, interferon and serum albumin, for the greater part of human origin. Some of these proteins have already proven their efficacy in human volunteers, however, their potential immunogenicity and their possible allergenic character still restrict their development. Several heterologous proteins have successfully been produced in plants. Among these proteins are monoclonal antibodies, hormones, vaccine antigens, enzymes and blood proteins (Dieryck et al., 1997; Florack et al., 1995; Ma et al., 1995) Matsumoto et al., 1163; Saito et al., 1991; Thanavala et al. 1995) A major limitation of plants, shared with other

heterologous expression systems like bacteria, yeast and insect cells, is their different glycosylation profile compared to mammals. In contrast to bacteria, having no N-linked glycans, and yeast, having only high mannose glycans, plants are able to produce proteins with complex N-linked glycans. Plant glycoproteins have complex N-linked glycans containing a α1,3 linked core fucose and β1,2 linked xylose residues not found in mammals (Lerouge et al., 1998) (figure 1). The core of plant N-glycans can, as in mammals, be substituted by 2 GlcNAc[1] residues, which are transferred by N-acetylglucosaminyltransferase I and II (Schachter, 1991) although their appearance varies (Rayon et al., 1999. N-glycans of some plant glycoproteins contain in addition a LewisA (Fucα1,4(Galβ1,3)GlcNAc) epitope (Fitchette Laine et al., 1997; Melo et al., 1997). However, plant glycoproteins lack the characteristic galactose (NeuAcα2,6Galβ1,4) containing complex N-glycans found in mammals, while also α1,6 linked core fucose is never found (figure 1; Schachter, 1991). A mouse monoclonal antibody produced in tobacco plants (Ma et al., 1995) has a typical plant N-glycosylation. 40% High-mannose glycans and 60% complex glycans containing xylose, fucose and 0, 1 or 2 terminal GlcNAc residues (Cabanes Macheteau et al., 1999).
In short, analyses of glycoproteins from plants have indicated that several steps in the glycosylation pathways of plants and mammals are very similar if not identical. There are however also clear differences, particularly in the synthesis of complex glycans. The complex glycans of plants are generally much smaller and contain beta-1,2 xylose or alpha-1,3 fucose residues attached to the Man3 (GlcNAc)2 core. Such residues on glycoprotein are known to be highly immunogenic. This will cause problems for certain applications of recombinant proteins carrying these sugars. In addition, although common and often essential on mammalian glycoproteins, sialic acid has never been found in plant

glycans. This is particularly relevant since experiments have shown, that the absence of terminal sialic acid on glycosidic side chains can dramatically decrease biological activity in vivo. Most likely, asialo-glycoprotein-receptors in the liver can bind to asialo-glycoprotein, and thereby cause a clearance of the glycoprotein from the circulation, which is reflected in a reduced metabolic half life and low bioactivity in vivo.

The invention provides a plant comprising a functional mammalian enzyme providing N-glycan biosynthesis that is normally not present in plants. It is especially the "plant" character of the glycans that makes glycoproteins produced in plants less suited for pharmaceutical use. This "plant" character imparts undesired antigenic and immunogenic characteristics to the glycoprotein in question, which would require a strategy intended to prevent immunogenicity of glycoproteins produced by transgenic plants. The aim of the strategy is to modify the genome of vegetable cells in such a manner that they ripen their proteins like human cells would. Numerous genes of glycosyl transferases of mammals have already been cloned, which is not the case in plants. In view of the ease of transformation of vegetable systems, the temptation is strong to "complement" the Golgi apparatus of plants by glycosyl transferases from mammals in order to "humanize" or "mammalize" the glycans of the glycoproteins they produce. The success of such a strategy is nonetheless not evident. In particular, the galactosylation and subsequent sialylation of recombinant glycoproteins in a vegetable cell depends not only on the transfer and the expression of the gene of the galactosyl and the sialyl transferase: these foreign enzymes must also be *active* in the vegetable cell, without detrimental effects to the plant cell, and last but not least, without detrimental effects to the transgenic plant as a whole.

To mammalise the glycosylation of plant for the production of tailor made glycoproteins in plants a xylosyltransferase and fucosyltransferase can be knocked out and at least one of several mammalian glycosyltransferases have to be expressed. Providing the xylosyltransferase and fucosyltransferase knock-outs and thereby reducing the unwanted glycosylation potential of plants is a feasible option because for example an *Arabidopsis thaliana* mutant mutated in the gene encoding N-acetylglucosaminyltransferase I was completely viable (Von Schaewen et al., 1993). As N-acetylglucosaminyltransferase I is the enzyme initiating the formation of complex glycans (Schachter, 1991), this plant completely lacks the xylose and fucose containing complex glycans.

In a preferred embodiment, the invention provides a plant comprising a functional (mammalian) protein, e.g. a transporter or an enzyme providing N-glycan biosynthesis that is normally not present in plants additionally comprising at least a second mammalian protein or functional fragment thereof that is normally not present in plants. It is provided by the invention to produce in plants a desired glycoprotein having a mammalian-type of glycosylation pattern, at least in that said glycoprotein is galactosylated. Again, desired glycoproteins may be any useful glycoprotein for which mammalian-like glycosylation is relevant.

In a preferred embodiment, the invention provides a plant according to the invention wherein said functional mammalian enzyme providing N-glycan biosynthesis that is normally not present in plants comprises (human) β1,4-galactosyltransferase. An important mammalian enzyme that is missing in plants is this β1,4-galactosyltransferase. cDNA's encoding this enzyme has been cloned from several mammalian species (Masri et al., 1988; Schaper et al., 1986). The enzyme transfers galactose from the activated sugar donor UDP-Gal in β1,4 linkage towards GlcNAc residues in N-linked

and other glycans (figure 1). These galactose residues have been show to play an important role in the functionality of e.g. antibodies (Boyd et al., 1995). β1,4-galactosyltransferase has recently been introduced in insect cell cultures (Hollister et al., 1998; Jarvis and Finn, 1996) to extend the N-glycosylation pathway of Sf9 insect cells in cell culture, allowing infection of these cultures with a baculovirus expression vector comprising a nucleic acid encoding a heterologous protein. It was shown that the heterologous protein N-linked glycans were to some extent more extensively processed, allowing the production of galactosylated recombinant glycoproteins in said insect cell cultures. Also the introduction of the enzyme into a tobacco cell suspension culture resulted in the production of galactosylated N-liked glycans (Palacpac et al., 1999) of endogenous proteins. However, no heterologous glycoproteins were produced in these plant cell cultures, let alone that such heterologous proteins would indeed be galactosylated in cell culture. Furthermore, up to date no transgenic plants comprising mammalian glycosylation patterns have been disclosed in the art. Many glycosylation mutants exist in mammalian cell lines Stanley and loffe, 1995; Stanley et al., 1996). However, similar mutations in complete organisms cause more or less serious malfunctioning of this organism (Asano et al., 1997; Herman and Hovitz, 1999; Loffe and Stanley, 1994). It is therefor in general even expected that β1,4-galactosyltransferase expression in a larger whole than cells alone (such as in a cohesive tissue or total organism) will also lead to such malfunctioning, for example during embryogenesis and/or organogenesis. Indeed, no reports have been made until now wherein a fully grown non-mammalian organism, such as an insect or a plant, is disclosed having the capacity to extend an N-linked glycan, at least not by the addition of a galactose. From many eukaryotic multicellular organisms, immortalized cell lines such as CHO,

Sf9 and hybridoma cell lines have been generated. These cell lines have been cultured for many generations, can carry many mutations and lack or have lost many characteristics which are essential for functioning of the intact organisms from which they are derived. To illustrate the latter, the fact that these immortalized cell lines can not be regenerated into complete intact organisms shows that important signaling pathways and components involved in cell-cell communication are lacking in these immortalized cell lines. It is known from literature that the N-linked glycosylation machinery of immortalized eukaryotic cell lines, such as CHO cells (Stanley and Loffe, 1995; Stanley et al., 1996) or Sf insect cell lines (Jarvis and Finn, 1996; Hollister et al., 1998), can be modified without having obvious negative effects on the viability of these cell lines, whereas in contrast similar mutations in complete organisms cause more or less serious malfunctioning of the organism (Aseno et al., 1997; Herman and Horvitz, 1999; Loffe and Stanley, 1994). Indeed no reports have been made that N-linked glycosylation can be extended, in such a way that N-linked glycans are formed that naturally do not occur, in eukaryotic cells which do have the potency to regenerate into viable organisms. Apparently, as compared to normal cells, immortalized cell lines are flexible and tolerant to new, not normal types of N-linked glycosylation but lack the capacity to develop into intact organisms.

Also modification of the N glycosylation machinery of immortalized tobacco BY2 cells has been reported. Introduction of GalT into this cell line results in the production of galactosylated N-linked glycans of endogenous proteins Palacpac et al., 1999). However, cells from this BY2 cell line can not be regenerated into viable tobacco plants. In addition and as described elsewhere in this patent application, the largest population was an abnormal hybrid type glycan (GlcNAc2Man5GlcNAcGal) suggesting premature

action of the introduced galactosyltransferase and an abnormal Golgi morphology and localisation of the galactosyltransferase in the BY2 cell line. This provides further evidence that this cell lines is significantly different from normal tobacco plant cells.
No reports have been made until now wherein a fully grown non-mammalian organism such as an insect or plant, is disclosed having the capacity to extend an N-linked glycan, at least not by the addition of a galactose.
Surprisingly, the invention now provides such a non-mammalian organism, a plant having been provided a a (functional) mammalian enzyme providing N-glycan biosynthesis that is normally not present in plants thereby for example providing the capacity to extend an N-linked glycan by the addition of a galactose. In a preferred embodiment, the invention provides such a plant wherein said enzyme shows stable expression. It is even provided that beyond said second mammalian protein a third mammalian protein is expressed by a plant as provided by the invention. The experimental part provides such a plant that comprises a nucleic acid encoding both an antibody light and heavy chain or (functional) fragment thereof. Of course, it is not necessary that a full protein is expressed, the invention also provides a plant according to the invention expression only a fragment, preferably a functional fragment of said second mammalian glycoprotein, said fragment having at least one activity of the whole protein and further being characterised by for example a truncated polypeptide chain, or a not fully extended glycan, for example only extended with galactose.
In a preferred embodiment, the invention provides a plant according to the invention wherein said second mammalian protein or functional fragment thereof comprises an extended N-linked glycan that is devoid of xylose and/or of fucose. As can be seen from figure 3 , plant-derived galactosylated glycoproteins still may contain xylose and fucose residues.

This in contrast to plant cell culture derived galactosylated glycoproteins (Palacpac et al., 1999) where these glycoproteins are essentially devoid of xylose and fucose residues. In plant cell cultures this is a result of the action of β1,4-galactosyltransferase on immature N-linked glycans, resulting in unnatural galactosylated 'hybrid type' N-linked glycans in which Golgi-mannosidase II and N-acetylglucosaminyltransferase II can not perform their function anymore. In a preferred embodiment, β1,4-galactosyltransferase is therefor expressed in plants in such a way that the enzyme acts in the Golgi apparatus on the natural substrates (figure 5). This means, after the action of N-acetylglucosaminyltransferase I, Golgi-mannosidase II and N-acetylglucosaminyltransferase II (and in plants, provided that these enzymes are not inhibited in another way, after or during the action of xylosyltransferase and fucosyltransferase). The present invention provides an plant in which galactosylation is essentially natural like it occurs in mammals.

The N-terminal cytoplasmic, transmembrane and stem region of glycosyltransferases determine the localisation of the enzyme in the ER or Golgy membrane. To provide natural or desirable glycosylation, glycosyltransferases can be expressed in plants as they occur in mammals, but can also be expressed as a fusion protein between two, or part of two, different glycosyltransferases. In this case the localisation is determined by one enzyme and the catalytic activity by a second enzyme. As example, a fusion between the cytoplasmic, transmembrane and stem region of plant xylosyltransferase and the catalitic domain of mammalian galactosyltransferase, providing an enzyme with galactosyltransferase activity and localisation of the xylosyltransferase.

If one would desire to further separate glycoproteins comprising extended N-linked glycan that is devoid of xylose and/or of fucose, or to produce these in a more purified way,

several possibilities are open. For one, several types of separation techniques exist, such as (immuno)affinity purification or size-exclusion chromatography or elecrtrophoresis, to mediate the required purification. Furthermore, another option is to use as starting material plants wherein the genes responsible for xylose and/or fucose addition are knocked-out.

In another embodiment, the invention provides a plant according to the invention wherein said N-linked glycan comprising galactose is further comprising sialic acid added thereto. In particular, the transfer of genes coding for sialyl transferases, enzymes which catalyze the addition of sialic acid on the glycan, into vegetable systems leads to even more stable glycoproteins during in vivo usage and hence better adapted to a possible therapy. The invention herewith provides the transfer of a sialic acid biosynthesis pathway to plants. In this invention when referring to plants the whole spectrum of plants ranging from algae to trees is intended unless otherwise specified. Plants in general lack sialic acid, a sugar residue needed for the enhanced function of certain glycoproteins like antibodies and hormones, in their N-linked glycans and also the substrates for sialylation have never been found. The invention provides plants that have the capacity to produce NeuAc containing N-linked glycans on their proteins. To establish this, up to 5 different heterologous genes are expressed in plants (see Table 1). To provide plants with the biosynthetic capacity to produce sialic acid, genes encoding up to five enzymes acting in the sialic acid biosynthesis pathway are transformed to plants. These enzymes from bacterial and mammalian origin are known: GlcNAc-2 epimerase, NeuAc synthase, CMP-NeuAc synthetase, CMP-NeuAc transporter and NeuAc transferase. All genes encoding the enzymes are if desired supplied with a (FLAG) tag to follow expression, and are transformed to e.g. tobacco and corn.

In another preferred embodiment, the invention provides a plant according to the invention wherein said N-linker glycan comprising galactose is further comprising or extended with glucoronic acid, glucoronyl, sulfate, sulfon, fucose, or other compound capable of extending galactose with linked to said galactose. This is particularly relevant since experiments have shown, that the absence of terminal sialic acid on glycosidic side chains can in general dramatically decrease biological activity <u>in vivo</u>. Most likely, asialo-glycoprotein-receptors in the liver can bind to asialo-glycoprotein, and thereby cause a clearance of the glycoprotein from the circulation, which is reflected in a reduced metabolic half life and low bioactivity <u>in vivo</u>. The presence of for example GlcA or another extending group but sialic acid has the same effect as the presence of sialic acid, it hinders the binding of a thus modified protein to the asialo-glycoprotein receptor of for example liver cells, thereby effectively increasing half-life, and thus clearance time, of such proteins, when used as therapeutic substance, i.e. as pharmaceutical composition. The invention thus provides an organism derived, herein in particular a plant-derived glycoprotein or functional fragment thereof comprising an extended N-linked glycan at least comprising galactose, said galactose further extended with a compound capable of extending galactose with, such as Glca to function in a similar way as silaic acid. For example, the invention provides plants that have the capacity to produce GlcA containing N-linked glycans on their proteins. To establish this, a gene encoding for example glucuronyltransferase (Terayama et al., PNAS 94:6093-6098, 1997) is expressed in plants according to the invention using methods known in the art or herein disclosed.

In this aspect, the invention is not limited to plants but also provides other organisms like animals, fungi or yeast, or cell lines like mammalian cell lines or insect cell lines

with the capacity to produce a glycoprotein (essentially non-sialiated) according to the invention wherein said N-linked glycan comprising galactose is further comprising or extended with for example glucuronic acid linked to galactose; which in essence has the same effect as the presence of sialic acid. The invention is not limited to extending the galactose by glucuronic acid which has the essentially the same effect as the presence of sialic acid in that it increase biological half-life and clearance time. Also sulfate, fucose or any other compound can be linked to galactose, thereby extending the carbohydrate group, by expressing a sulfotransferase, fucosyltransferase or other enzyme that transfers sulfate, fucose or other compound to galactose residues can be used to increase half-life. The invention thus provides a method to increase half-life or improve clearance time of a pharmaceutical composition comprising as active component a glycoprptein, comprising providing said glycoprotein with a compound, attached to galactose, that replaces or provides sialic acid function and thus provides at least reduced reactivity with a asialo-glycoprotein-receptor, preferably wherein said receptor is at least present on a liver-cell. Also more than one compound can be transferred to galactose, for example glucuronic acid that is extended by sulfate by expressing a sulfotransferase that transfers sulfate to glucuronic acid. The invention is not limited to those cases in which extension of galactose by other compounds than sialic acid has the same effect as extension with sialic acid. Extension of galactose by other compounds than sialic acid can have a function by its own for example in interaction with other compounds, cells or organisms. Furthermore, it has the advantage that components, otherwise extended by sialic acid, but now for example with glucoronic acid, or sulfate of fucose groups, for that matter, can easily be recognised and thus distinguished from like endogenous compounds extended with sialic acid. For example,

a pharmaceutical composition comprising a glycosylated protein, such as a glycoprotein hormone, or erytrhopoetin (EPO), normally provided with sialic acid, but now with for example a sulfon, or with glucoronic acid, can easily be recognised, facilitating detection of the foreign compounds. As an example, figure 6 shows that tobacco plants that express human β1,4 galactosyltransferase and rat β1,3 glucuronyltransferase form the desired structure GlcAβ1,Gal on their glycoproteins as is clearly shown by the binding of a specific antibody (mouse monoclonal antibody 412) to GlcAβ 1,Gal structure.

Extending galactose with other compounds than silalic acid can also have advantages for the production of recombinant proteins in plants. It can make the glycoprotein or glycan of the glycoprotein more stable by preventing galactosydases and/or other glycosydases from degrading the N-glycan. It can, by doing that, increase the galactosylation. It can also be of use in a purification procedure, for example by facilitating affinity purification by specific antibodies, lectins or other compounds. if desired, the compound by which galactose is extended or further comprised can, after purification of the recombinant glycoprotein, be removed, by for example a specific glycosydase, sulfatase, phosphatase, or other suitable enzyme.

In another preferred embodiment, the invention provides a plant according to the invention wherein said N-linked glycan comprising galactose is further comprising other sugar residues not directly linked to galactose, for example core alpha1,6 linked fucose or beta1,4- or beta1,6 linked N-acetylglucosamine (GlcNAc). To establish this, a gene or genes encoding for example core alpha1,6 fucosyltransferase or/and GlcNAc-transferase III, GlcNAc-transferase IV, GlcNAc-transferase V and/or GlcNAc-transferase VI are expressed in plants according to the invention using methods known in the art or herein disclosed.

In general, herein is provided a method to tailor N-linked glycosylation for the production of heterologous glycoproteins in plant species with typical plant like glycosylation patterns similar to those as shown in figure 1, i.e. which lack the typical mammalian proteins involved in N-linked glycosylation such as, but not limited to, beta1-4 galactosyltransferases and glucoronyl transferases. Generating stably transformed plants which produce tailored glycoproteins with commercial interest can be established by inoculating plant cells or tissues with Agrobacterium strains containing a (binary) vector which comprises both nucleotide sequences encoding N-glycosylation modifying enzymes and genes encoding commercially interesting heterologous glycoproteins. Alternatively, stably transformed plants which produce tailored glycoproteins with commercial interest can be generated by simultaneous inoculation (co-transformation) of two or more Agrobacterium strains each carrying a vector comprising either nucleotide sequences encoding N-glycosylation modyfying enzymes or nucleotide sequences encoding glycoproteins of commercial interest. Alternatively, stably transformed plants which produce tailored glycoproteins with commercial interest can be generated by (multiple) crossing(s) of plants with modified N-glycosylation with plants which express nucleotide sequences encoding proteins of commercial interest. In all of these procedures, the vector may also comprise a nucleotide sequence which confers resistance against a selection agent. In order to obtain satisfactorily expression of the proteins involved in N-glycosylation and of the glycoproteins or polypeptides of commercial interest, the nucleotide sequences may be adapted to the specific transcription and translation machinery of the host plant as known to people skilled in the art. For example, silent mutations in the coding regions may be introduced to improve codon usage and specific promoters

may be used to drive expression of the said genes in the relevant plant tissues. Promoters which are developmentally regulated or which can be induced at will, may be used to ensure expression at the appropriate time, for example, only after plant tissues have been harvested from the field and brought into controlled conditions. In all these cases, choice of expression cassettes of the glycosylation modifying proteins and of the glycoproteins of commercial interest should be such that they express in the same cells to allow desired post translational modifications to the said glycoprotein.

In the detailed description the invention provides a plant as defined herein before according to the invention which comprises a tobacco plant, or at least a plant related to the genus *Nicotiana*, however, use for the invention of other relatively easy transformable plants, such as *Arabidopsis thaliana*, or *Zea mays*, or plants related thereto, is also particularly provided. For the production of recombinant glycoproteins, use of duckweed offers specific advantages. The plants are in general small and reproduce asexually through vegetative budding. Nevertheless, most duckweed species have all the tissues and organs of much larger plants including roots, stems, flowers, seeds and fronds. Duckweed can be grown cheaply and very fast as a free floating plant on the surface of simple liquid solutions from which they can easily be harvested. They can also be grown on nutrient-rich waste water, producing valuable products while simultaneously cleaning wastewater for reuse. Particularly relevant for pharmaceutical applications, duckweed can be grown indoors under contained and controlled conditions. Stably transformed Duckweed can for example be regenerated from tissues or cells after (co)-inoculating with Agrobacterium strains containing each a (binary) vector which comprises one or more nucleotide sequences of interest encoding N-glycosylation modifying

enzymes and/or genes encoding commercially interesting heterologous glycoproteins. The duckweed plant may for example comprise the genus Spirodella, genus Wolffia, genus Wolffiella, or the genus Lemna, Lemna minor, Lemna miniscula and Lemna gibba.
Expression in tomato fruits also offers specific advantages. Tomatoes can be easily grown in greenhouses under contained and controlled conditions and tomato fruit biomass can be harvested continuously throughout the year in enormous quantities. The watery fraction containing the glycoproteins of interest can be readily separated from the rest of the tomato fruit which allows easier purification of the glycoprotein. Expression in storage organs of other crops including but not limited to the kernels of corn, the tubers of potato and the seeds of rape seed or sunflower are also attractive alternatives which provide huge biomass in organs for which harvesting and processing technology is in place.

Herewith, the invention provides a method for providing a transgenic plant, such as transgenic *Nicotiana*, *Arabidopsis thaliana*, or corn, potato, tomato, or duckweed , which are capable of expressing a recombinant protein, with the additional desired capacity to extend an N-linked glycan with galactose comprising crossing said transgenic plant with a plant according to the invention comprising at least one functional mammalian protein, e.g. a transporter or an enzyme providing N-glycan biosynthesis that is normally not present in plants, harvesting progeny from said crossing and selecting a desired progeny plant expressing said recombinant protein and expressing a functional (mammalian) enzyme involved in mammalian-like N-glycan biosynthesis that is normally not present in plants. In a preferred embodiment, the invention provides a method according to the invention further comprising selecting a desired progeny plant expressing said recombinant protein comprising an extended N-

linked glycan et least comprising galactose. In the detailed description a further description of a method according to the invention is given using tobacco plants and crossings thereof as an example.

With said method as provided by the invention, the invention also provides a plant expressing said recombinant protein and expressing a functional (mammalian) enzyme involved in mammalian-like N-glycan biosynthesis that is normally not present in plants. Now that such a plant is provided, the invention also provides use of a transgenic plant to produce a desired glycoprotein or functional fragment thereof, in particular wherein said glycoprotein or functional fragment thereof comprises an extended N-linked glycan et least comprising galactose.

The invention additionally provides a method for obtaining a desired glycoprotein or functional fragment thereof comprising for example an extended N-linked glycan at least comprising galactose comprising cultivating a plant according to the invention until said plant has reached a harvestable stage, for example when sufficient biomass has grown to allow profitable harvesting, followed by harvesting said plant with established techniques known in the art and fractionating said plant with established techniques known in the art to obtain fractionated plant material and at least partly isolating said glycoprotein from said fractionated plant material. In the detailed description (see for example figure 4) is further explained that an antibody having been provided with an extended N-linked glycan at least comprising galactose is provided.

The invention thus provides a plant-derived glycoprotein or functional fragment thereof comprising an extended N-linked glycan at least comprising galactose, for example obtained by a method as explained above. Such a plant-derived glycoprotein with an extended glycan at least comprising galactose essentially can be any desired glycoprotein that

can be expressed in a plant. For example, antibodies, FSH, TSH and other hormone glycoproteins, other hormones like EPO, enzymes like antitrypsine or lipase, cellular adhesion molecules like NCAM or collagen can be produced in plants and be provided with essentially mammalian glycosylation patterns. Expression of such proteins can be performed by using a method known in the art. For example, by stable expression via Agrobacterium mediated transformation, electroporation or particle bombardment, but also by transient expression using a virus vector like PVX or other method, glycosyltransferases or an other protein extending glycan biosynthesis, and/or said glycoprotein could be expressed under control of a specific promoter to facilitate expression in certain tissues or organs.
Herewith, the invention also provides use of such a plant-derived glycoprotein or functional fragment thereof according to the invention for the production of a pharmaceutical composition, for example for the treatment of a patient with an antibody, a hormone, a vaccine antigen, an enzyme, or the like. Such a pharmaceutical composition comprising a glycoprotein or functional fragment thereof is now also provided. The invention is further explained in the detailed description without limiting it thereto.

Detailed description

One important enzyme involved in mammalian N-glycan biosynthesis that is not present in plants is β1,4-galactosyltransferase. Here, for one, the stable expression of β1, 4-galactosyltransferase in tobacco plants is described. The physiology of these plants is not obviously changed by introducing β1,4-galactosyltransferase and the feature is inheritable. Crossings of a tobacco plant expressing β1,4-galactosyltransferase with a plant expressing

the heavy and light chain of a mouse antibody produced antibody having terminal galactose in similar amounts as hybridoma produced antibodies. Herein it is thus shown that the foreign enzyme can be successfully introduced in plants. A clear increase in galactose containing glycoproteins is observed. Moreover, this feature is inheritable and there is no visible phenotypical difference between the galactosyltransferase plants and wild type. A mouse monoclonal antibody produced in these plants has a degree of terminal galactoses comparable to hybridoma produced antibody. This shows that not only endogenous proteins become galactosylated but also a recombinantly expressed mammalian protein.

Materials and Methods

Plasmids and plant transformation

A plant transformation vector containing human β1,4-galactosyltransferase was constructed as follows: a 1.4 kb BamHI/XbaI fragment of pcDNAI-GalT (Aoki et al., 1992; Yamaguchi and Fukuda, 1995) was ligated in the corresponding sites of pUC19. Subsequently, this fragment was re-isolated using surrounding KpnI and HincII sites and cloned into the KpnI and SmaI site of pRAP33 (named pRAP33-HgalT). Using AscI and PacI sites the CaMV35S promotor-cDNA-Nos terminator cassette of pRAP33-HgalT was cloned in the binary vector pBINPLUS (van Engelen et al., 1995). Modifications to the published protocol are: After incubation with *A. tum.*, leaf discs were incubated for three days in medium containing 1 mg/ml of NAA and 0.2 mg/ml BAP and the use of 0.25 mg/ml cefotaxime and vancomycine to inhibit bacterial growth in the callus and shoot inducing medium. 25 rooted shoots were transformed from <u>in vitro</u> medium to soil and, after several weeks, leaf material of these plants was analysed.

Northern blotting

The β1,4-galactosyltransferase RNA level in the transgenic plants was analyzed by northern blotting (Sambrook et al., 1989) RNA was isolated from leafs of transgenic and control plants as described (De Vries et al., 1991). Ten μg of total RNA was used per sample. The blot was probed with a [^{32}P]dATP labeled SstI/XhoI fragment, containing the whole GalT cDNA, isolated from pBINPLUS-HgalT.

Glycoprotein analysis

Total protein extracts of tobacco were prepared by grinding leafs in liquid nitrogen. Ground material was diluted 10 times in SDS page loading buffer (20 mM of This-HCl pH 6.8, 6% glycerol, 0.4% SDS, 20 mM DTT, 2.5 ìg/ml Bromophenol Blue). After incubation at 100°C for 5 min insoluble material was pelleted. Supernatants (12.5 μl/sample) were run on 10% SDS-PAGE and blotted to nitrocellulose. Blots were blocked overnight in 0.5% Tween-20 in TBS and incubated for 2 hours with peroxidase conjugated RCA_{120} (Ricinus Communis Agglutinin, Sigma) (1 μg/ml) in TBS-0.1% tween-20. Blots were washed 4 times 10 minutes in TBS-0.1% tween-20 and incubated with Lumi-Light western blotting substrate (Roche) and analysed in a lumianalyst (Roche). A rabbit polyclonal antibody directed against Horseradish peroxidase (HRP, Rockland Immunochemicals) was split in reactivity against the xylose and fucose of complex plant glycans by affinity chromatography with bee venom phospholipase according to (Faye et al., 1993). A rabbit anti LewisA antibody was prepared as described (Fitchette Laine et al., 1997). Blots were blocked with 2% milkpowder in TBS and incubated in the same buffer with anti-HRP, anti-xylose, anti-fucose or anti-Lewis-A. As secondary antibody alkaline HRP-conjugated sheep-anti-mouse was used and detection was as described above.

Plant crossings

Mgr48 (Smant et al., 1997) is a mouse monoclonal IgG that has been expressed in Tobacco plants. The construct used for transformation was identical to monoclonal antibody 21C5 expressed in tobacco (van Engelen et al., 1994). Flowers of selected tobacco plants with high expression of β1,4-galactosyltransferase were pollinated with plants expressing Mgr48 antibody. The F1 generation was seeded and plants were screened for leaf expression of antibody by western blots probed HRP-conjugated sheep-anti-mouse and for galactosyltransferase expression by RCA as described above.

Purification of IgG1 from tobacco

Freshly harvested tobacco leaves were ground in liquid nitrogen. To 50 g of powdered plant material, 250 ml of PBS, containing 10 mM $Na_2S_2O_5$, 0.5 mM EDTA, 0.5 mM PMSF and 5 g polyvinylpolypyrrolid, was added. After soaking for 1 hour (rotating at 4°C), insoluble material was removed by centrifugation (15 min, 15,000g, 4°C). The supernatant was incubated overnight (rotating at 4°C) with 1 ml of proteinG-agarose beads. The beads were collected in a column and washed with 10 volumes of PBS. Bound protein was eluted with 0.1 M glycine pH 2.7 and immediately brought to neutral pH by mixing with 1 M Tris pH 9.0 (50 μl per ml of eluate). Purified antibody was quantified by comparison of the binding of HRP-conjugated sheep-anti-mouse to the heavy chain on a western blot with Mgr48 of known concentration purified from hybridoma medium (Smant et al., 1997).

Hybridoma Mgr48 and plant produced Mgr48 was run on 10% SDS-PAGE and blotted as described above. Detection with RCA was as described above. For antibody detection, blots were probed with HRP-conjugated sheep-anti-mouse and detected with Lumi-Light western blotting substrate as described above.

Results

Human β1,4-galactosyltransferase galactosylates endogenous proteins in Nicotiana tobacum.

Human β1,4-galactosyltransferase (Masri et al., 1988) was introduced in tobacco plants by Agrobacterium mediated leaf disk transformation of plasmid pBINPLUS-HgalT containing a cDNA that includes a complete coding sequence. Twenty-five plants selected for kanamicin resistance were analysed for mRNA levels by northern hybridization (fig 2A). The same plants were analyzed by the galactose binding lectin RCA_{120} (Ricinus Cummunis Agglutinin). RCA binds to the reaction product of β1,4-GalT (Galβ1,4GlcNAc) but also to other terminal β linked galactose residues. RCA binds to one or more high molecular weight proteins isolated from non transgenic control tobacco plants (fig 2B). Probably these are Arabinogalactan or similar proteins. RCA is known to bind to Arabinogalactan proteins (Schindler et al., 1995). In a number of the plant transformed with Human β1,4-galactosyltransferase, in addition, binding of RCA to a smear of proteins is observed. This indicates that in these plants many proteins contain terminal β linked galactose residues. There is a good correlation between the galactosyltransferase RNA expression level and the RCA reactivity of the trangenic plants. Human β1,4-galactosyltransferase expressed in transgenic plants is therefor able to galactosylate endogenous glycoproteins in tobacco plants.
As it is known that galactosylated N-glycans are poor acceptors for plant xylosyl- and fucosyltransferase (Johnson and Chrispeels, 1987), the influence of expression of β1,4-galactosyltransferase on the occurrence of the xylose and fucose epitope was investigated by specific antibodies. A polyclonal rabbit anti-HRP antibody that reacts with both the xylose and fucose epitope shows a clear difference in binding

to isolated protein from both control and transgenic plants (figure 3).

Recombinantly produced antibody is efficiently galactosylated.

The effect of expression of β1,4-galactosyltransferase on a recombinantly expressed protein was investigated. Three tobacco plants expressing β1,4-galactosyltransferase (no. GalT6, GalT8 and GalT15 from fig. 2) were selected to cross with a tobacco plant expressing a mouse monoclonal antibody. This plant, expressing monoclonal mgr48 (Smant et al., 1997), was previously generated in our laboratory. Flowers of the three plants were pollinated with mgr48. Of the F1 generation 12 progeny plants of each crossing were analysed for the expression of both antibody and β1,4-galactosyltransferase by the method described in materials and methods. Of crossing GalT6xmgr48 and GalT15xmgr48 no plants were found with both mgr48 and GalT expression. Several were found in crossing GalT8xmgr48. Two of these plants (no.11 and 12), were selected for further analysis.
Using proteinG affinity, antibody was isolated from tobacco plants expressing mgr48 and from the two selected plants expressing both mgr48 and β1,4-galactosyltransferase. Equal amounts of isolated antibody was run on a protein gel and blotted. The binding of sheep-anti-mouse-IgG and RCA to mgr48 from hybridoma cells, tobacco and crossings GalT8xmgr48-11 and 12 was compared (figure 4). Sheep-anti-mouse-IgG bound to both heavy and light chain of all four antibodies isolated. RCA, in contrast, bound to hybridoma and GalT plant produced antibody but not to the antibody produced in plants expressing only mgr48. When the binding of sheep-anti-mouse-IgG and RCA to the heavy chain of the antibody is quantified, the relative reaction of RCA (RCA binding / sheep-anti-mouse-IgG binding) to GalT8xmgr48-11 and 12 is respectively 1.27

and 1.63 times higher than the ratio of hybridoma produced antibody. This shows that RCA binding to the glycans of antibody produced in GalT plants is even higher than to hybridoma produced antibody. Although the galactosylation mgr48 from hybridoma is not quantified, this is a strong indication that the galactosylation of antibody produced in these plants is very efficient.

Construction of plant expression vectors with cDNA's encoding α 2,6 sialytransferase, β 1,3-glucuronyltransferase and β 1,4-galactosyltransferase.

The available β 1,4-galactosyltransferase vector was not in a suitable format to easily combine with α2,6-sialyltransferase and β 1,3-glucuronyltransferase clones. Therefore, by using PCR, the coding region of β 1,4-galactosyl-transferase cDNA, α 2,6-sialyltransferase cDNA and β 1,3-glucuronyl-transferase cDNA have been cloned in plant expression vectors. Constructs are made in which galactosyltransferase is combined with either sialyltransferase or glucuronyltransferase in one vector, in order to enable simultaneous expression of the enzymes in transgenic plants after only one transformation. The galactosyltransferase expression is controlled by the 35S promoter, whereas expression of sialyltransferase and glucuronyltransferase is controlled by the 2'promoter.

There is a need for an accessible and standardised source of FSH for therapeutic and diagnostic purposes, which is guaranteed to be free of LH activity.
FSH preparations normally are derived from ovine or porcine pituitaries, which always implies the presence of (traces of) LH, and the risk of contamination with prion-like proteins. Substitution of brain derived FSH for plant produced

recombinant FSH may be a good method of eliminating these problems. However, production of bioactive animal glycoproteins in plants, especially for therapeutic purposes, requires modification of plant-specific sugar sidechains into a mammalian type of glycans. The invention provides recombinant bFSH by infecting stably transformed tobaccoplants capable of forming mammalian type of glycans, with recombinant Tobacco Mosaic Virus TMV containing the genes for bFSH or bFSHR.

Construction of single chain (sc) bFSH into pKS (+) bluescript vector, construction of sc-bFSH-TMV and sc-bFSH-HIS-TMV

In order to circumvent the need of simultaneous expression of the two separate genes of bFSH-alpha and bFSH-beta subunits in plants, we decided to construct a bFSH fusion gene. By overlap PCR we fused the carboxyl end of the beta subunit to the amino end of the alpha subunit (without a linker). In addition, we constructed a second sc-bFSH version carrying a 6x HIS tag at the C-terminus of the alpha subunit, which will allow us to purify the recombinant protein from the plant. Both, sc-bFSH and sc-bFSH-HIS constructs were subcloned into the cloning vector pKS(+) bluescript. The correctness of the clones was confirmed by sequence analysis. Sc-bFSH was subcloned into the TMV vector. Two positive clones were chosen to make *in vitro* transcripts and Inoculate N. *Bentahamiana* plants. After a few days, plants showed typical viral infection symptoms, which suggested the infective capacity of the recombinant TMV clones. In order to test whether the sc-bFSH RNA is stably expressed in systemically infected leaves, 8 days post inoculation RNA was isolated from infected *N. benthamiana* leaves and a reverse transcriptase polymerase chain reactions using bFSH specific primers was performed. In all cases we obtained a PCR fragment of the expected size, indicating the stability of

our Sc-bFSH-TMV construct. Extracts of infected plants are used for Western blot analyses and ELISA to determine whether Sc-bFSH is expressed and folded properly.

Abbreviations used:
GlcNAc, N-Acetylglucosamine; Fuc, fucose; Gal, galactose; GalT, â1,4-galactosyltransferase; RCA, Ricinus Cummunis Agglutinin; Tables

Tables

Table 1: Enzymes of sialic acid biosynthesis pathway

No	enzyme	Catalysed reaction	localisation	origin
1	GlcNAc-2 epimerase	GlcNAc←→ManNAc	cytoplasm	pig
2	NeuAc synthase	ManNAc + PEP ←→ NeuAc	cytoplasm	Clostridium
3	CMP-NeuAc synthetase	NeuAc + CMP → CMP-NeuAc	nucleus	mouse
4	CMP-NeuAc transporter	Cytoplasm → Golgi lumen	Golgi membrane	mouse
5	NeuAc transferase	CMP-NeuAc + Gal-R → NeuAc-Gal-R + CMP	Golgi	human
	Gal transferase	UDP-Gal + GlcNac-R → Gal-GlcNAc-R + UDP	Golgi	human

Figure legends

Figure 1

Major differences between mammalian and plant complex N-linked glycans. Drawn are typical N-linked glycans. Numerous variations, both extended or truncated, occur in mammals and plants.

Figure 2

Comparison of RNA levels and product of ß1,4-galactosyltransferase. Upper panel: Northern blot of total RNA isolated from 25 transgenic plants, including a not transformed control plant (0), detected with a human ß1,4-galactosyltransferase probe. Lower panel: Western blot of the same plant probed with RCA to detect terminal galactose residues on glycoproteins. M. indicates the molecular weight marker.

Figure 3

Western blot showing the binding of lectin and antibody to protein isolated from wild-type and a ß1,4-galactosyltransferase plant (no.8 from figure 2). A: RCA as in figure 2, B: anti HRP (detecting both xylose and fucose) antibody, C: anti xylose antibody, D: anti fucose antibody.)

Figure 4

Western blot showing RCA and sheep-anti-mouse-IgG binding to purified antibody produced in hybridoma culture (Hyb), tobacco plants (plant) and tobacco plants co-expressing ß1,4-galactosyltransferase (GalT11 and GalT12). H.C.: heavy chain, L.C. light cain.

Figure 5

Tobacco cell cultures expressing galactosyltransferase produce unnatural hybrid N-glycans while tobacco plants expressing galactosyltransferase have natural, mammalian like

galactosylation. To get natural galactosylation, galactosyltransferase should act after mannosidase II and GlcNAcTransferase II.

Figure 6

Western blot showing the expression of GlcAβ1,3Gal structure in transgenic tobacco by binding of an antibody (412) directed against the glucuronic acid-galactose (GlcAβ1,3Gal) stucture to protein isolated from 8 plants expressing human β1,4 galactosyltransferase and rat β1,3 glucuronyltransferase and a wildtype controll plant (-).

References

Aoki, D., Lee, N., Yamaguchi, N., Dubois, C., and Fukuda, M. N. (1992). Golgi retention of a trans-Golgi membrane protein, galactosyltransferase, requires cysteine and histidine residues within the membrane-anchoring domain. Proceedings Of The National Academy Of Sciences Of The United States Of America 89, 4319-4323.

Asano, M., Furukawa, K., Kido, M., Matsumoto, S., Umesaki, Y., Kochibe, N., and Iwakura, Y. (1997). Growth retardation and early death of beta- 1,4-galactosyltransferase knockout mice with augmented proliferation and abnormal differentiation of epithelial cells. Embo j 16, 1850-7.

Boyd, P.N., Lines A.C., and Patel A.K. (1995). The effect of the removal of sialic acid, galactose and total carbohydrate on the functional activity of Campath-1H. Mol immunol 32, 1311-8.

Cabanes Macheteau, M., Fichette Laine, A.C., Loutelier Bourhis, C., Lange, C., Vine, N.D., Ma, J.K., Lerouge, P., and Faye, L. (1999). N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology 9, 365-72.

De Vries, S., Hoge, H., and Bisseling, T. (1991). Isolation of total and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, B. Gelvin, R.A. Schilperoort and D.P.S. Verma, eds. (Dordrecht: Kluwer Academic Publishers), pp. B6/1-13.

Dieryck, W., Pagnier J., Poyart, C., Marden, M.C., Gruber, V., Bournat, P., Baudino, S., and Merot, B. (1997). Human haemoglobin from transgenic tobacco [letter] Nature 386, 29-30.

Faye, L., Gomord, V., Fitchette Laine, A.C. and Chrispeels, M.J. (1993). Affinity purification of antibodies specific for Asn-linked glycans containing alpha 1-->3 fucose or beta 1-->2 xylose. Anal Biochem 209, 104-8.

Fichette Laine, A.C., Gomord, V., Cabanes, M., Michalski, J.C., Saint Macary, M., Foucher, B., Cavelier, B., Hawes, C., Lerouge, P., and Faye, L. (1997). N-glycans harboring the Lewis a epitope are expressed at the surface of plant cells. Plant J 12, 1411-7.

Florack, D., Allefs, S., Bollen, R., Bosch, D., Visser, B., and Stiekema, W. (1995). Expression of giant silkmoth cecropin B genes in tobacco. Transgenic Research 4, 132-141.

Herman, T., and Horvitz, H.R. (1999). Three proteins involved in Caenorhabditis elegans vulval invagination are similar to components of a glycosylation pathway. Proc Natl Acid Sci U S A 96, 979-9.

Hollister, J.R., Shaper, J.H. and Jarvis, D.L. (1998). Stable expression of mammalian beta, 1,4-galactosyltransferase extends the N-glycosylation pathway in insect cells. Glycobiology 8, 473-80.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S. G., and Fraley, R.T. (1985). A simple and general method for transferring genes into plants. Science, USA 227, 1229-1231.

Ioffe, E., and Stanley, P. (1994). Mice lacking N-acetylglucosaminyltransferase I activity die at mid-gestation, revealing and essential role for complex or hybrid N-linked carbohydrates. Proc Natl Acid Sci U S A 91, 728-32.

Jarvis, D.L., and Finn, E.E. (1996). Modifying the insect cell N-glycosylation pathway with immediate early baculovirus expression vectors. Nat Biotechnol 14, 1288-92.

Jenkins, N. Parekh, R.B., and James D.C. (1996). Getting the glycosylation right: implications for the biotechnology industry. Nat Biotechnol 14, 975-81.

Johnson, K.D., and Chrispeels, M.J. (1987). Substrate specificities of N-acetylglucosaminyl-, fucosyl-, and xylosyltransferases that modify glycoproteins in the Golgi apparatus of bean cotyledons. Plant Physiology 84, 1301-1308.

Lerouge, P., Cabanes Macheteau, M., Rayon, C, Fischette Laine, A.C., Gomord, V, and Faye, L. (1998). N- glycoprotein

biosynthesis in plants: recent developments and future trends. Plant Mol Biol 38, 31-48.

Ma, J.K., Hiatt, A., Hein, M., Vine, N.D., Wang, F., Stabila, P., van Dolleweerd, C., Mostov, K. and Lehner, T. (1995). Generation and assembly of secretory antibodies in plants [see comments.] Science 268, 716-9.

Masri, K.A., Appert, H.E., and Fukuda, M.N. (1988). Identification of the full-length coding sequence for human galactosyltransferase (beta-N-acetylglucosaminide: beta 1,4-galactosyltransferase). Biochem Biophys Res Commun 157, 657-63.

Matsumoto, S., Ikura, K., Ueda, M., and Sasaki, R. (1163). Characterization of a human glycoprotein (erythropoietin) produced in cultured tobacco cells. Plant Molecular Biology 27, 1163-1172.

Melo, N.S., Nimtz, M., Conradt, H.S., Fevereiro, P.S., and Costa, J. (1997). Identification of the human Lewis(a) carbohydrate motif in a secretory peroxidase from a plant cell suspension culture (Vaccinium myrtillus L.). FEBS Lett 415, 186-91.

Palacpac, N.Q., Kimura, Y., Fuijyama, K., Yoshida, T., and Seki, T. (1999). Structures of N-linked oligosaccharides of glycoproteins from tobacco BY2 suspension cultured cells. Biosci Biotechnol Biochem 63, 35-9.

Palacpac, N.Q., Yoshida, S., Sakai, H., Kimura, Y., Fuijyama, K., Yoshida, T., and Seki, T. (1999). Stable expression of human beta 1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. Proc Natl Acad Sci U S A 96, 4692-7.

Rayon, C., Cabanes Macheteau, M., Loutelier Bourhis, C., Salliot Maire, I., Lemoine, J., Reiter, W.D. Lerouge, P., and Faye, L. (1999). Characterization of N-glycans from Arabidopsis. Application to a fucose-deficient mutant. Plant Physiol 119, 725-34.

Saito, K., Noji, M. Ohmori, S., Imai, Y., and Murakoshi, I. (1991). Integration and expression of a rabbit liver

cytochrome P-450 gene in transgenetic Nicotiana tabacum. Proceedings Of The National Academy Of Sciences Of The United States Of America 88, 7041-7045.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual (Plainview, NY: Cold Spring Harbor Lab. Press).

Schachter, H. (1991). The `yellow brick road' to branched complex N-glycans. Glycobiology 1, 453-61.

Schindler, T., Bergfeld, R., and Schopfer, P. (1995). Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extention growth. Plant JU 7, 25-36.

Schaper, N.L., Shaper, J.H., Meuth, J.L., Fox, J.L., Chang, H., Kirsch, I.R. and Hollis, G.F. (1986). Bovine galactosyltransferase: identification of a clone by direct immunological screening of a cDNA expression library. Proc Natl Acad Sci U S A 83, 1573-7.

Smant, G., Goverse, A., Stokkermans, J.P.W.G., De Boer, J.M., Pomp, H., Zilverentant, J.F. Overmars, H.A. Helder, J., Schots, A. and Baaker, J. (1997) Potato root diffusate-induced secretion of soluble, basic proteins originating from the subventral esophageal glands of potato cyst nematodes. Phytopathology 87, 839-845.

Stanley, P., and loffe, E, (1995). Glycosyltransferase mutants: key to new insights in glycobiology. Faseb j 9, 1436-44.

Stanley, P., Raju, T.S., and Bhaumik, M. (1996). CHO cells provide access to novel N-glycans and developmentally regulated glycosyltransferases. Glycobiology 6, 695-9.

Thanavala, Y., Yang, Y.F., Lyons, P., Mason, H.S., and Arntzen, C. (1995). Immunogenicity of transgenetic plant-derived hepatitis B surface antigen. Proceedings of the National Academy of Sciences of the United States of America 92, 3358-3361.

van Engelen, F.A., Molthoff, J.W., Conner, A.J., Nap, J.P., Pereira, A., and Stiekema, W.J. (1995). pBINPLUS: an

improved plant transformation vector based on pBIN19. Transgenetic Res 4 , 288-90.

van Engelen, F.A., Schouten, A., Molthoff, J.W., Roosein, J. Salinas, J., Dirkse, W.G., Schots, A., Bakker, J., Gommers, F.J., Jongsma, M.A., and et al. (1994). Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenetic tobacco. Plant Mol Biol. 26, 1701-10.

von Schaewen, A., Sturm, A., O'Neill, J., and Chrispeels, M.J. (1993) Isolation of a mutant Arabidopsis plant that lacks N-acetyl glucosaminyl transferase I and is unable to synthesize Golgi-modified complex N-linked glycans. Plant Physiol 102, 1109-18.

Yamaguchi, N., and Fukuda, M.N. (1995). Golgi retention mechanism of beta-1,4-galactosyltransferase. Membrane-spanning domain-dependent homodimerization and association with alpha- and beta-tubulins. J Biol Chem 270, 12170-6.

Claims

1. A plant comprising a functional protein such as a transporter or a (mammalian) enzyme or functional fragment thereof providing N-glycan biosynthesis.

2. A plant according to claim 1 additionally comprising at least a second (mammalian) protein or functional fragment thereof.

3. A plant according to claim 1 or 2 wherein said enzyme comprises (human) β1,4-galactosyltransferase.

4. A plant according to claim 2 wherein said second mammalian protein comprises an antibody chain.

5. A plant according to claim 2 wherein said second mammalian protein or functional fragment thereof comprises an extended N-linked glycan.

6. A plant according to claim 4 or 5 wherein said second mammalian protein or functional fragment thereof is stabily expressed.

7. A plant according to claim 5 or 6 wherein said N-linked glycan comprises galactose.

8. A plant according to anyone of claims 5 to 7 wherein said second mammalian protein or functional fragment thereof comprises an extended N-linked glycan that is devoid of xylose.

9. A plant according to anyone of claims 6 to 8 wherein said second mammalian protein or functional fragment thereof comprises an extended N-linked glycan that is essentially devoid of fucose.

10. A plant according to anyone of claims 7 to 9 wherein said N-linked glycan comprising galactose further comprises sialic acid.

11. A plant according to anyone of claims 7 to 9 wherein said N-linked glycan comprising galactose further comprises glucoronyl.

12. A plant according to anyone of claims 1 to 11 which comprises a tobacco plant.

13. A method for providing a transgenic plant capable of expressing a recombinant protein with the capacity to extend an N-linked glycan with galactose comprising crossing said transgenic plant with a plant according to claim 1, harvesting progeny from said crossing and selecting a desired progeny plant expressing said recombinant protein and expressing a functional (mammalian) enzyme involved in (mammalian) N-glycan biosynthesis that is normally not present in plants.

14. A method according to claim 13 further comprising selecting a desired progeny plant expressing said recombinant protein comprising an extended N-linked glycan at least comprising galactose.

15. A method according to claim 13 or 14 wherein said plant comprises a tobacco plant.

16. A plant obtainable by a method according to anyone of claims 13 to 15.

17. Use of a plant according to claim 2 or 16 to produce a desired glycoprotein or functional fragment thereof.

18. Use according to claim 17 wherein a said glycoprotein or functional fragment thereof comprises an extended N-linked glycan at least comprising galactose.

19. A method for obtaining a desired glycoprotein or functional fragment thereof comprising cultivating a plant according to claim 2 or 16 until said plant has reached a harvestable stage, harvesting and fractionating said plant to obtain fractionated plant material and at least partly isolating said glycoprotein from said fractionated plant material.

20. A plant-derived glycoprotein or functional fragment thereof comprising an extended N-linked glycan at least comprising galactose.

21. A plant-derived glycoprotein or functional fragment thereof obtained by a method according to claim 19.

22. Use of a glycoprotein or functional fragment thereof according to claim 20 or 21 for the production of a pharmaceutical composition.

23. A pharmaceutical composition comprising a glycoprotein or functional fragment thereof according to claim 20 or 21.

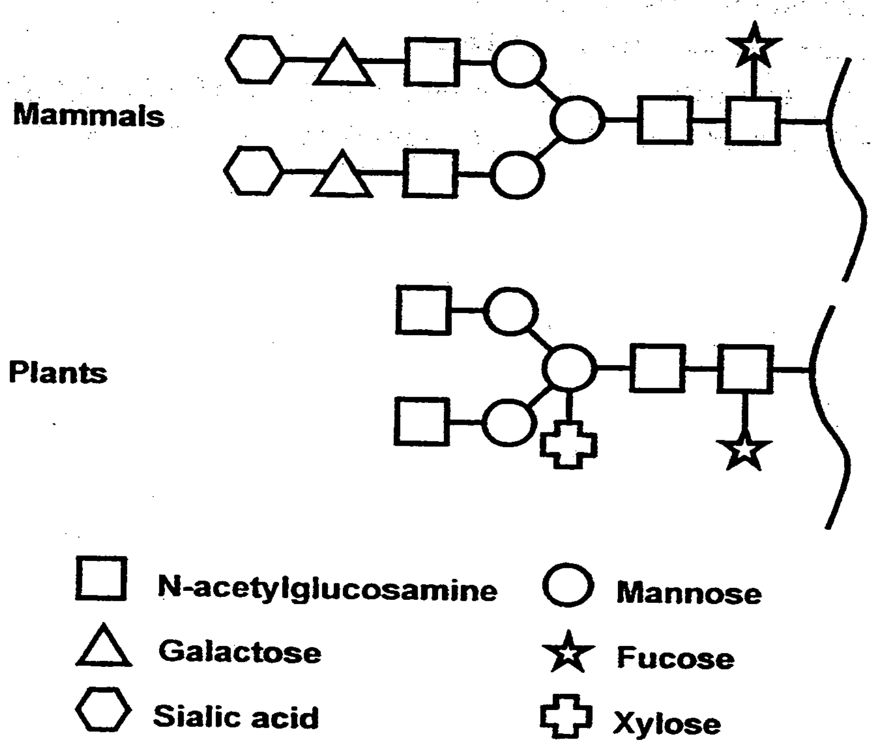
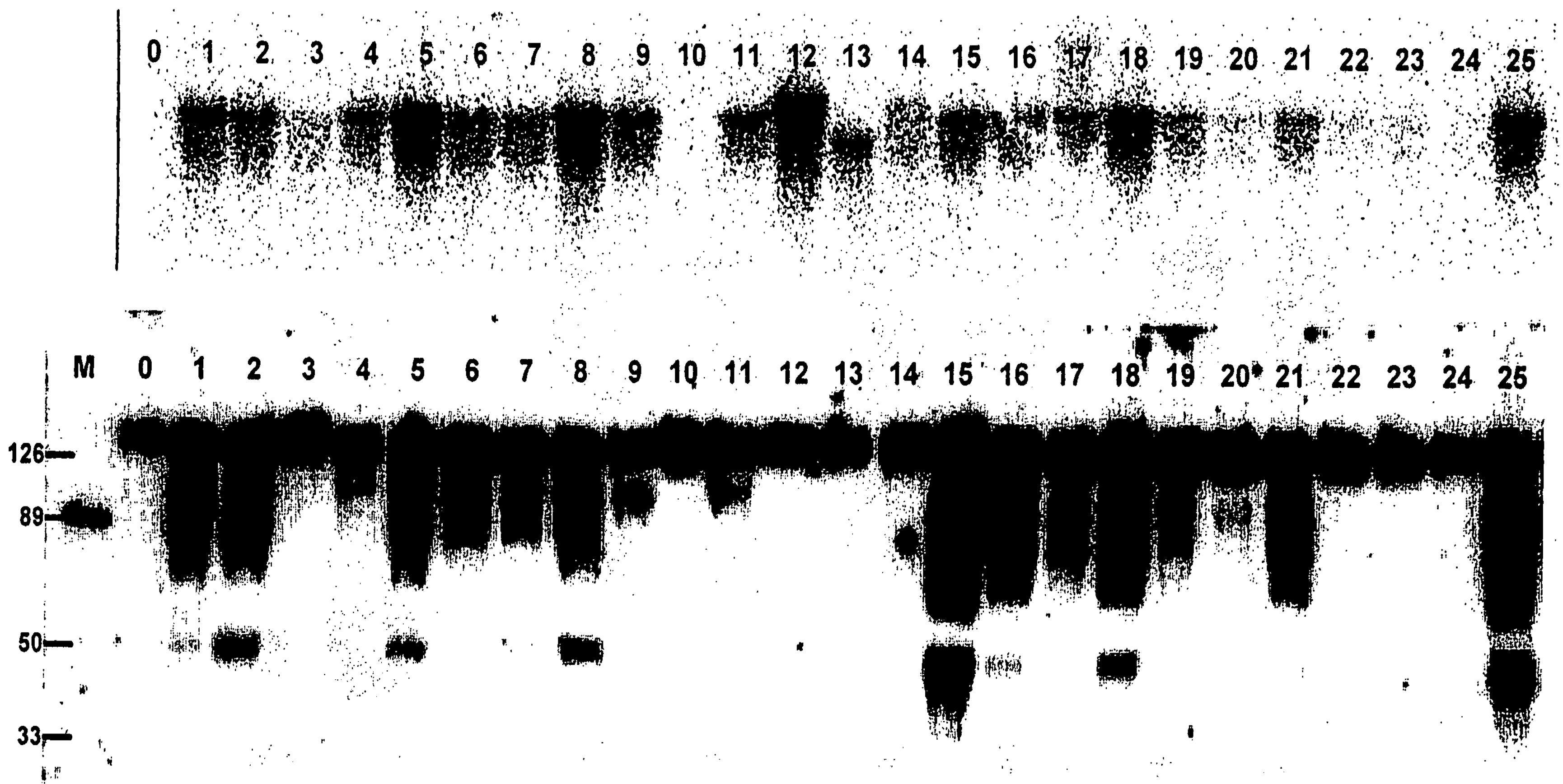

Mammals
Plants
N-acetylglucosamine
Mannose
Galactose
Fucose
Sialic acid
Xylose

Fig. 1



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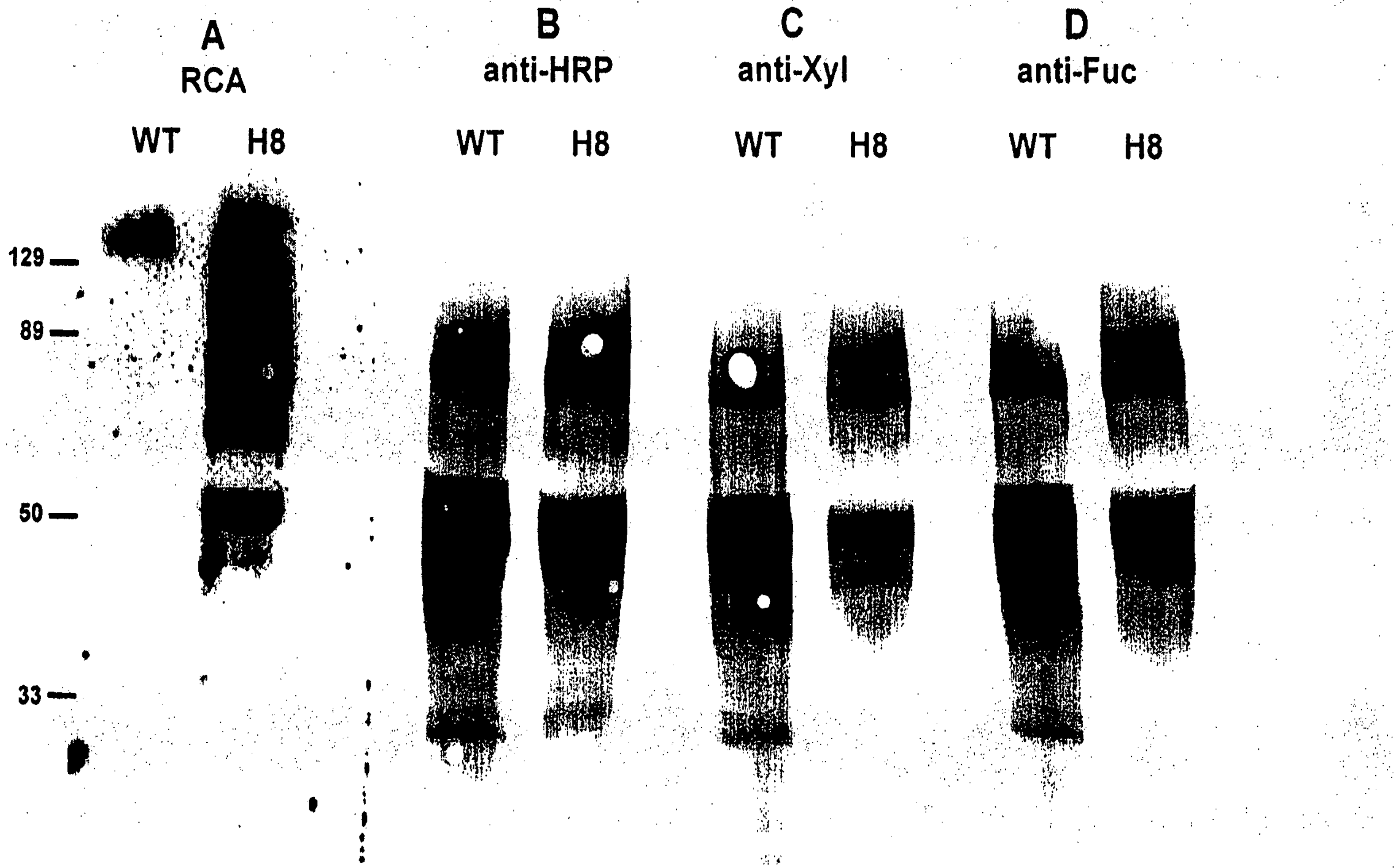

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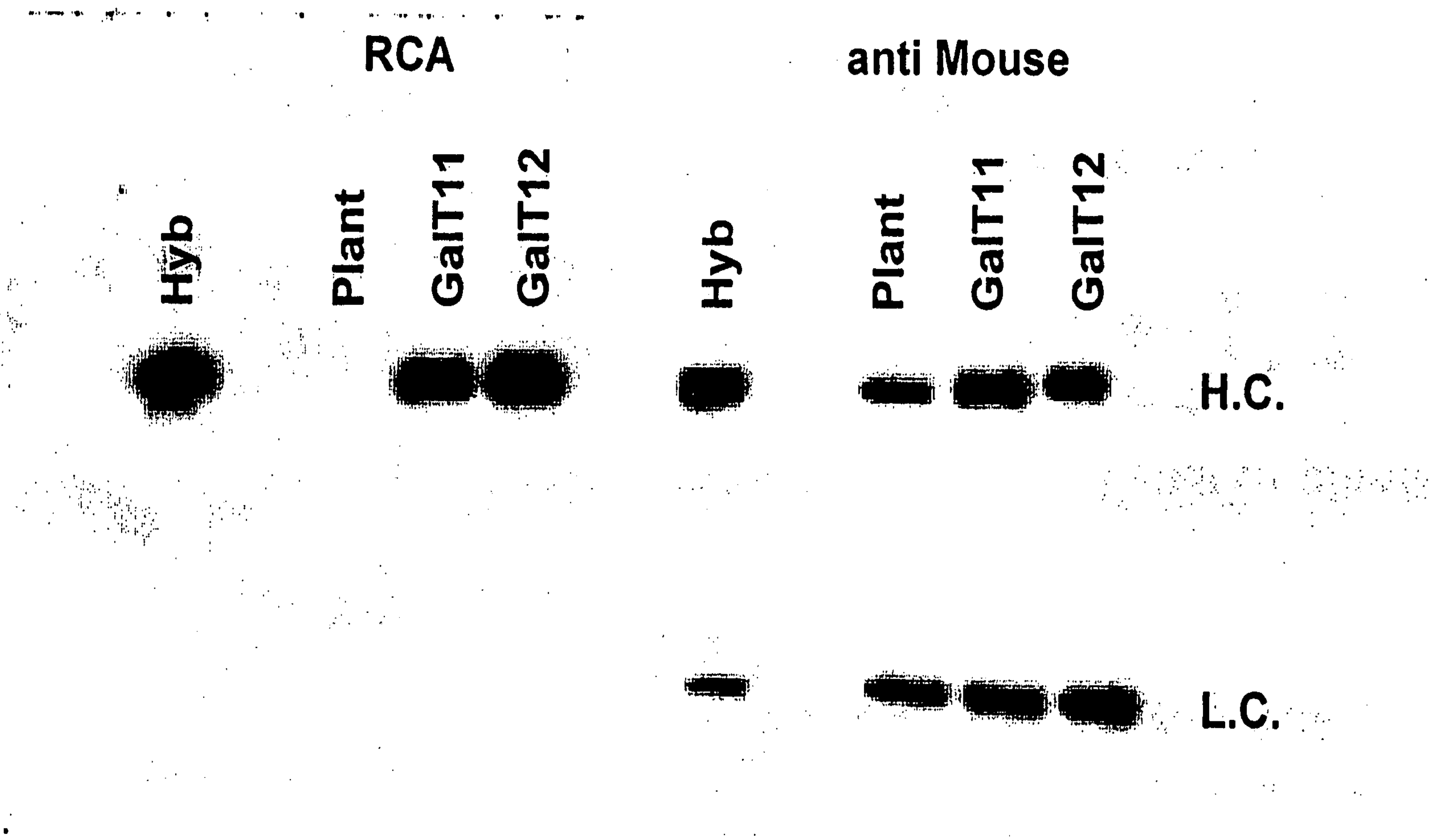

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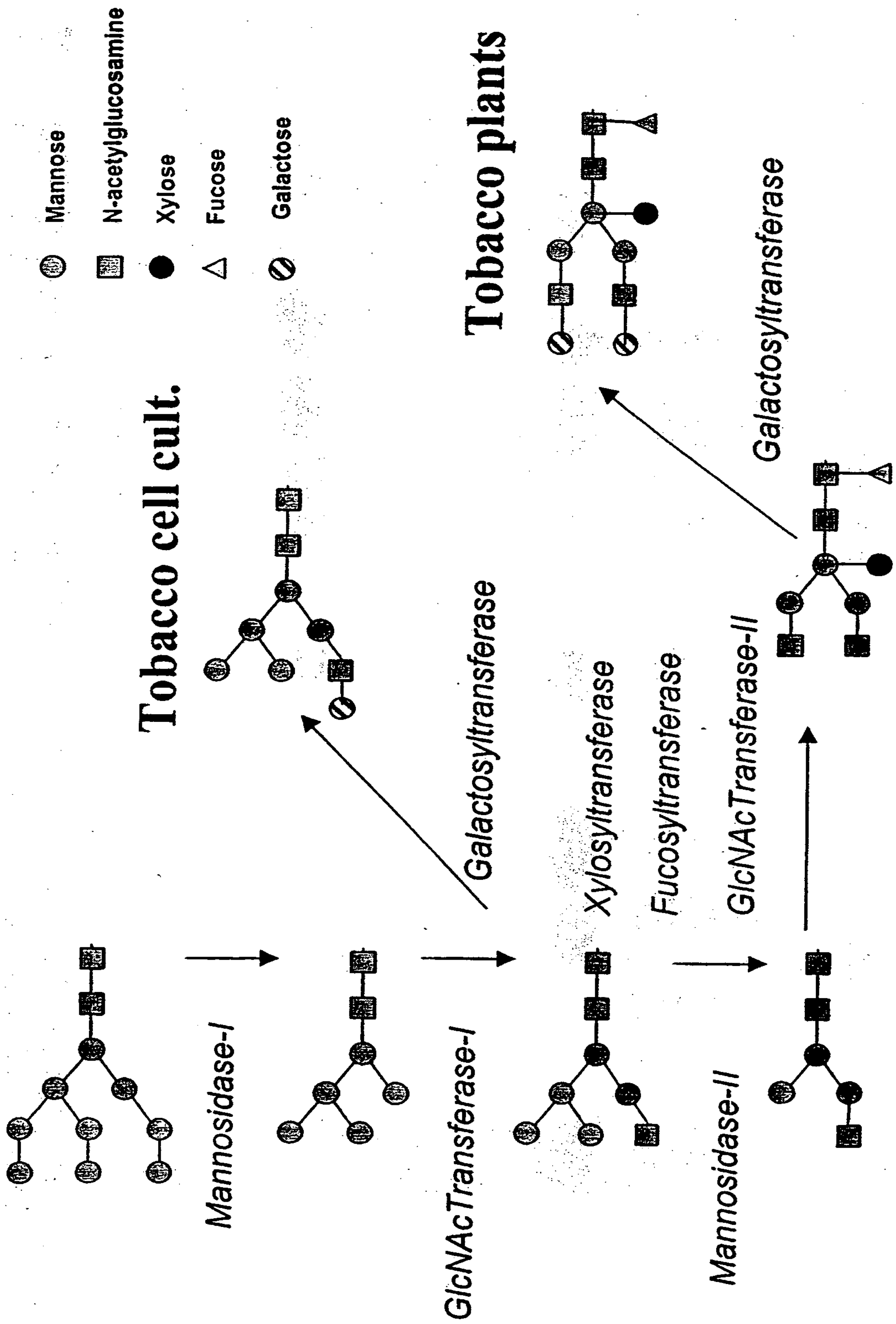

figure 5. Tobacco cell cultures expressing galactosyltransferase produce unnatural hybrid N-glycans while tobacco plants expressing galactosyltransferase have natural, mammalian like galactosylation. To get natural galactosylation, galactosyltransferase should act after mannosidase II and GlcNAcTransferase II.

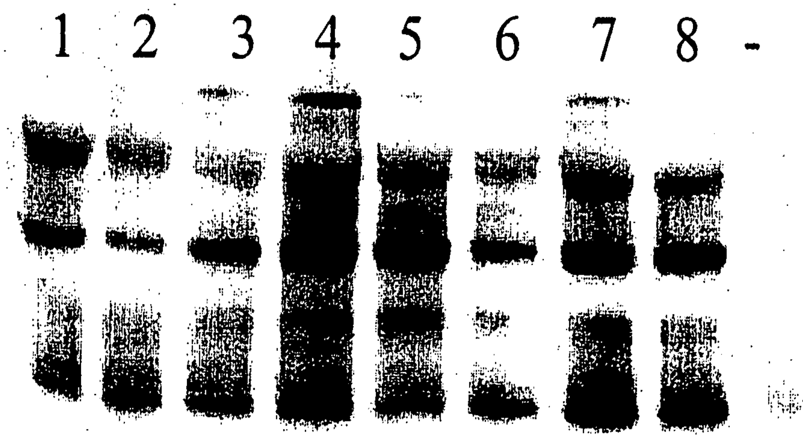

figure 6. Western blot showing the expression of GlcAβ1,3Gal structure in transgenic tobacco by binding of an antibody (412) directed against the glucuronic acid-galactose (GlcAβ1,3Gal) structure to protein isolated from 8 plants expressing human β1,4 galactosyltransferase and rat β1,3 glucuronyltransferase and a wildtype controll plant (-).

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00775

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/10 C07K14/47 A61K38/00 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, PAJ, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PALACPAC NQ. ET AL.: "Stable expression of human beta1,4-galactosyltransferase in plant cells difies N-linked glycosylation patterns." PROC NATL ACAD SCI U S A 1999 APR 13;96(8):4692-7, XP002133652 cited in the application page 4692, right-hand column	1-3,5-9, 12,17-23
X	WO 99 51185 A (INCYTE PHARMA INC ;PATTERSON CHANDRA (US); CORLEY NEIL C (US); GUE) 14 October 1999 (1999-10-14) page 25, line 21 - line 30	1,3

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2001	30/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEE EG. ET AL.: "Targeting of active sialyltransferase to the plant Golgi apparatus." PLANT CELL 1998 OCT;10(10):1759-68, XP002133653 the whole document	1
X	WO 99 29879 A (VON SCHAEWEN ANTJE) 17 June 1999 (1999-06-17) page 23 -page 24	1,2,5,6, 8,9,12, 17-23
X	GOMEZ L AND CHRISPEELS M J: "Complementation of an Arabidopsis thaliana mutant that lacks complex asparagine-linked glycans with the human cDNA encoding N-acetylglucosaminyltransferase I" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 91, no. 91, March 1994 (1994-03), pages 1829-1833-1833, XP002100921 ISSN: 0027-8424 the whole document	1
A	JENKINS N. ET AL.: "Getting the glycosylation right: impications for the biotechnology industry." NAT BIOTECHNOL 1996 AUG;14(8):975-81, XP002133654 the whole document	
A	VAN ENGELEN F A ET AL: "Coordinate expression of antibody subunit genes yields high levels of functional antibodies in roots of transgenic tobacco" PLANT MOLECULAR BIOLOGY,NL,NIJHOFF PUBLISHERS, DORDRECHT, vol. 26, no. 26, 1994, pages 1701-1710-1710, XP002122098 ISSN: 0167-4412 cited in the application the whole document	

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KLEENE R ET AL: "EXPRESSION OF SOLUBLE ACTIVE HUMAN BETA1, 4 GALACTOSYLTRANSFERASE IN SACCHAROMYCES CEREVISIAE" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS,US,ACADEMIC PRESS INC. ORLANDO, FL, vol. 201, no. 1, 30 May 1994 (1994-05-30), pages 160-167, XP000608638 ISSN: 0006-291X the whole document ---	
A	WRIGHT A ET AL: "Effect of glycosylation on antibody function: implications for genetic engineering" TRENDS IN BIOTECHNOLOGY,GB,ELSEVIER PUBLICATIONS, CAMBRIDGE, vol. 15, no. 1, 1 January 1997 (1997-01-01), pages 26-32, XP004016809 ISSN: 0167-7799 page 30, right-hand column -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte. .onal Application No
PCT/NL 00/00775

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9951185 A	14-10-1999	US 5955282 A	21-09-1999
		AU 3210299 A	25-10-1999
		EP 1067893 A	17-01-2001
WO 9929879 A	17-06-1999	DE 19754622 A	10-06-1999
		AU 2268899 A	28-06-1999
		EP 1038014 A	27-09-2000